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Interaction of Blood Proteins With Solid Surfaces

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ABSTRACT

The adsorption of blood proteins on surfaces has been investigated in order to develop a detailed understanding of the initial series of events that occur when a synthetic material is implanted in the cardiovascular system. The overall objective of the investigation is to help provide a rational basis for the characterization and design of materials and the development of test methods. The relationship between surface potential and protein-surface interaction was investigated quantitatively by in situ ellipsometry to determine the molecular extension and adsorbance of fibrinogen, serum albumin, and γ-globulin on platinum as a function of impressed surface potential. For all three proteins, no change in adsorbance from the value at rest potential occurred as the surface potential was progressively made more anodic until a critical potential was attained, at which time the adsorbance increased significantly. The changes in extension observed as a result of changes in induced surface potential indicated, however, that conformational changes in the adsorbed layer were occurring as a result of surface potential. The determination of the bound fraction (fraction of carbonyl groups directly in contact with the surface) and extension of adsorbed γ-globulin and β-lactoglobulin as a function of solution concentration indicate conformational changes with surface population. Similar measurements on γ-globulin crosslinked prior to adsorption indicate that the native conformation exists at low surface coverage. Investigation of the rates of desorption of albumin from silica into buffer indicates a fast initial desorption followed by a considerably slower desorption removing most, but not all, of the adsorbed protein during the time periods investigated.

INTERACTION OF BLOOD PROTEINS WITH SOLID SURFACES

SUMMARY

The adsorption of blood proteins on surfaces has been investigated in order to develop a detailed understanding of the initial series of events that occur when a synthetic material is implanted in the cardiovascular system. The overall objective of the investigation is to help provide a rational basis for the characterization and design of materials and the development of test methods.

The blood proteins investigated, fibrinogen, prothrombin, γ-globulin, and serum albumin, have been selected either because of implication in the clotting process or occurrence in high concentration in blood plasma. Special attention has been given to the purity of these materials and additional repurification has been carried out in our laboratory when necessary. The major emphasis in this investigation has been the molecular conformational changes occurring upon interaction of the protein with the surfaces.

The relationship between surface potential and protein-surface interaction was investigated by quantitative in situ ellipsometry to determine the molecular extension and adsorbance of fibrinogen, serum albumin, and γ-globulin on platinum as a function of impressed surface potential. For all three proteins, as the surface potential was progressively made more anodic, no change in adsorbance from the value at rest potential occurred until a critical potential was attained, at which the adsorbance increased significantly. The changes in extension observed as a result of changes in induced surface potential indicated, however, that conformational changes in the adsorbed layer were occurring as a result of surface potential.

The previous measurement of the bound fraction (fraction of carbonyl groups of an adsorbed protein molecule directly in contact with the surface) of prothrombin and serum albumin on a silica surface showed that approximately ten percent of these groups were attached to the surface and that this number was independent of concentration or time. Additional studies have now been made with γ-globulin and β-lactoglobulin which show a change in bound fraction and extension as a function of solution concentration for these two proteins, inferring a conformational change with surface population. Further investigations with prior cross-linked γ-globulin, to decrease any conformational changes that may occur upon adsorption, indicate that the native conformation exists at low surface coverage.

Work has been initiated to obtain bound fraction measurement on a hydrophobic surface. Techniques are being developed and some results are given.

Rates of desorption of albumin from silica have been measured into water and buffer. In both cases there is a fast initial desorption. In the case of buffer, this is followed by a second, much slower desorption rate, which removes most, but not all of the adsorbed protein.

The radiolabeled albumin was used to investigate the apparent porous nature of a low temperature isotropic carbon surface. High protein concentrations in small areas of the surface indicated surface scratches or cracks.

INTERACTION OF BLOOD PROTEINS WITH SOLID SURFACES

INTRODUCTION

The initial event or series of events that occur when a synthetic material is implanted in the cardio-vascular system is the adsorption of components in the blood on the surface of that material. The adsorption of blood factors and proteins on the surface of the implant can modify both their biological activity (1,2) and the subsequent interaction of formed cellular elements (3). It is the intent of this investigation to develop a detailed understanding of the interaction of four major blood proteins with surfaces in order to help provide a rational basis for the design of materials and test methods.

A more extensive rationale for this investigation was given in a previous report (4), together with a detailed description of three major techniques employed: ellipsometry, infrared difference spectroscopy, and radiotracer techniques. The work has been expanded during this reporting period to investigate the possible relationship between surface potential and thrombosis, the dependence of protein conformation on surface concentration, and the correlation between ellipsometric and bound fraction measures of protein conformation. In addition, both ion beam deposited (IBD) and low temperature isotropic (LTI) carbon were investigated using ellipsometric and radiotracer techniques.

EXPERIMENTAL

Protein Purity

Fibrinogen

Bovine and human fibrinogen are repurified in our laboratories by the Laki (5) and Batt (6) methods, respectively, to yield a product that is greater than 96.7% clottable. (The theoretical maximum is 97%.)

Albumin

The human and bovine serum albumin are obtained as four times recrystallized material with a purity of 100%, as determined by electrophoretic analysis. This material is used as received.

Prothrombin

Cohn Fraction III has been used for most of the work reported here as well as in our previous report (4). The results on this material have been compared with an extremely highly purified prothrombin that was kindly supplied to us by Professor Craig Jackson, Washington University. He has determined the purity by a chromatographic separation and found that there is no detectable thrombin activity.

γ-Globulin

This material is recrystallized and has a purity greater than 95% as determined by electrophoretic analysis. It is used as received.

β-Lactoglobulin

Bovine β-lactoglobulin, three times recrystallized, was used as received.

Surface Potential

A suitable cell for ellipsometric studies at controlled potentials was constructed by removing the bottom of a fused silica optical cell and replacing it with a sample holder which allowed a 2 x 5 cm specimen to be clamped to the base of the cell. A liquid-tight seal was made by a butyl rubber gasket. Electrical contact with the working electrode was accomplished by pressing a silver strip against the back of the specimen. The counter electrode was formed by a 16 gauge platinum wire spiral separated from the solution within the cell by a fine fritted glass disc. A saturated calomel reference electrode, maintained in a separate compartment, was connected to the cell by an agar-KCl salt bridge. All adsorption experiments were carried out at 37°C.

The surface used in all experiments reported here was bright platinum cleaned in boiling 1:1 concentrated HNO_3-H_2SO_4 and heated at 500°C. The specimen was quenched in 0.15N NaCl solution, following which the cell was assembled and filled with 0.15N NaCl as quickly as possible. Human fibrinogen and serum albumin, as well as bovine

γ-globulin, were obtained from Nutritional Biochemical Co.* All solutions were prepared using 0.15N NaCl and the pH was adjusted to 7.4.

The procedure utilized for making the ellipsometric measurements have been described previously (4, 7). In the experiments reported here, the protein was allowed to adsorb for approximately 120 minutes with no impressed potential. At this time, the most negative potential of those to be impressed was applied and ellipsometric readings were taken. The potential was then incremented in the positive direction at approximately 30 minute intervals, and and the procedure was repeated until a critical potential, characteristic of the particular protein, was reached. The data were analyzed using an interative method (8) to determine both the extension and the amount adsorbed.

Fraction of Free Amino Groups Blocked by DEM

The concentration and number of free amino groups blocked by the cross-linking reaction with diethyl malonimidate dihydrochloride (DEM) can be determined in a straightforward (9) manner whenever the extinction coefficient at 280nm is unchanged as a result of the reaction. Reaction of DEM with serum albumin changed its extinction coefficient. However, since the protein could be purified by dialysis vs. distilled water and the purified material lyophilized and subsequently redissolved, gravimetric techniques could be utilized to determine the extinction coefficient. In the case of γ-globulin, however, dialysis vs. distilled water results in precipitation and loss of the protein. We have, therefore, utilized ultraviolet optical density, Kjeldahl nitrogen analysis, and visible optical density following reaction with ninhydrin for the cross-linked γ-globulin to determine simultaneously its fraction of nitrogen, extinction coefficient at 280nm, and the fraction of free amino groups blocked. These parameters are all interrelated, since the introduction of an unknown number of cross-links results in a change of both the fraction of nitrogen and the extinction coefficient.

The resolution of this problem requires the formulation and solution of three simultaneous equations describing the situation. The extinction coefficient at 280nm, ε_{CL}, is given by

$$\varepsilon_{CL} = \frac{A}{L(N/f_{NCL})} \qquad (1)$$

*Certain commercial materials and instruments are identified here and elsewhere in this publication in order to adequately specify the experimental materials and procedures. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the equipment or instruments identified are necessarily the best available for the purpose.

where A is the optical density at 280nm of a protein solution subsequently used for Kjeldahl analysis, L the path length, N the value (in mg N_2/ml protein solution) from Kjeldahl analysis of cross-linked γ-globulin, and f_{NCL} the fraction of nitrogen in the cross-linked protein. The value of f_{NCL} is calculated from

$$f_{NCL} = \frac{M_p f_N + 14\ R\ F_B}{M_p + M_{CL}(R/2)F_B} \tag{2}$$

where M_p is the molecular weight of the protein (∿150,000 for γ-globulin), f_N the fraction of nitrogen in native material, R the total number of replaceable amino groups in the protein (120 for γ-globulin), and M_{CL} the molecular weight of the cross-link introduced (66 for DEM). R/2 is the maximum number of cross-links possible, while F_R is the actual fraction of free amino groups blocked by the cross-linking reagent. From the reaction of ninhydrin (9) with both native and cross-linked protein, the fraction of amino groups blocked is

$$F_B = \frac{A_N^{570}\left(\frac{A_{CL}^{280}/\varepsilon_{CL}}{A_N^{280}/\varepsilon_N}\right) - A_{CL}^{570}}{A_N^{570}\left(\frac{A_{CL}^{280}/\varepsilon_{CL}}{A_N^{280}/\varepsilon_N}\right)} \tag{3}$$

where A_N^{570} and A_{CL}^{570} are the optical densities at 570nm of the native and cross-linked protein following reaction with ninhydrin, A_N^{280} and A_{CL}^{280} are the optical densities at 280nm of the native and cross-linked protein solutions used in ninhydrin reaction, and ε_N, is the extinction coefficient of the native protein at 280nm. For the cross-linked γ-globulin prepared for this study of adsorbed conformation, the simultaneous solution of Eqs. 1-3 yielded values of ε_{CL} = 1.611, f_{NCL} = 0.1672, and F_B = 0.555.

Radiotracer

The radiotracer experiments utilized the same platinum, carbon, and similarly prepared polyethylene as used for the ellipsometry studies. ^{131}I labeled human albumin was obtained from Squibb, and small samples of serum albumin and fibrinogen incorporating acetyl groups labeled with tritium were obtained from Dr. Israel Miller of the Weizmann Institute. Isotropic carbon heart valve discs were supplied to us by General Atomic Corp. Lot no. 21 C-163 which was used for radiotracer studies had a nominal 10%

silicon content present as micron sized particles of silicon carbide. The linear polyethylene, a well characterized polymer, Standard Reference Material 1475, was obtained from the National Bureau of Standards. The quartz and platinum slides were cleaned with hot 1:2 concentrated HNO_3:H_2SO_4 for one hour, heat treated at 500°C for ten minutes, and placed in buffer while still hot. They were never subsequently exposed to air. The carbon and polyethylene samples were cleaned by refluxing in a soxlet for several days, first with ethanol and then water.

Solutions of the unlabeled protein were made up by weight in pH 7.4 phosphate buffer and ^{131}I labeled protein was added by syringe. Adsorption was carried out in a covered glass vessel equipped with a magnetic stirrer and placed in a thermostatically controlled water bath maintained at 37 $\pm$ .1 °C. Desorption was carried out on slides on which maximum adsorbance was attained under the adsorption conditions, as determined by separate experiments. After adsorption was carried out on a group of slides, they were divided into two sets. One set was rinsed, dried and counted in order to exactly determine the initial amount of labeled protein adsorbed. The remaining set was rinsed and transferred, without drying, into a thermostated vessel containing solvent. After a predetermined desorption time, the slides were removed, rinsed, dried, and counted. The rate of exchange of adsorbed labeled protein with unlabeled protein in solution was measured in a similar manner, except that the slides were transferred to a vessel containing unlabeled protein of the same solution concentration.

The carbon samples were examined to determine the distribution of labeled protein remaining after cleaning procedures were applied. After adsorption with HSA-^{131}I the samples were scrubbed with detergent solution, rinsed and dried. Autoradiographic film was applied, and after suitable periods of exposure (about 2 months) the films were developed and stripped from the surface.

All counting of ^{131}I labeled samples was done in a low background beta counter with a plastic window. Tritium labeled samples were counted without a window. A mask was used in order to maintain consistent counting geometry, define the counted area, and eliminate edge effects. In order to establish the specific activity of the labeled protein solutions, 5 and 10µl aliquots of the solution were taken and evaporated to dryness on stainless steel planchets. Separate experiments established that the quartz and chrome samples had the same backscatter as the steel while the platinum showed 25% more backscatter. The activities were adjusted for this difference.

RESULTS AND DISCUSSION

MOLECULAR EXTENSION

Surface Potential

The possible relationship between surface potential and thrombosis on synthetic implants has been explored by Sawyer and associates (e.g., see ref. 10). The dependence of protein adsorption on surface potential has been investigated by others using capacitance techniques (11), cyclic voltammetry (12), potentiostatic and qualitative ellipsometric techniques (13), and more recently, internal reflection spectroscopy (14). We have utilized quantitative *in situ* ellipsometric techniques to simultaneously determine both the molecular extension and adsorbance for purified blood proteins as a function of surface potential.

As shown in Figures 1-3, which are typical of the many experiments performed, fibrinogen, serum albumin, and γ-globulin all exhibited a critical potential at which significant enhanced adsorption on platinum at pH 7.4 compared to the value at rest potential. In each case, the adsorbance was unchanged from the rest potential value as the clamped potential became more anodic, until the critical potential was reached. At this point, the rate of enhanced adsorption became approximately proportional to the surface potential. A set of parameters resulting from the collected data of a number of experiments are shown in Table I.

These results of enhanced adsorbance are in accord with the qualitative ellipsometric (13) and capacitance (11) measurements, as well as the more direct internal reflection methods (14).

The conformation of all three proteins initially adsorbed at rest potential was changed by the applied surface potentials. In the case of fibrinogen and γ-globulin, there was a sharp increase in extension when the most cathodic potential of the range shown in Table I was applied. Subsequent experiments with γ-globulin established that the increase in extension was an equilibrium property and not just a transient one following the initiation of the surface potential experiment. As the potential became more anodic, the extension in both cases decayed to smaller values. The serum albumin film decreased markedly in thickness from 10 to 4nm when the critical potential of +0.6V/SCE was applied, resulting in a more compact film, as judged by a concomitant increase in the index of refraction.

Carbon

Ion Beam Deposited

Several Ion Beam Deposited (IBD) carbon samples from Whittaker Corp. were examined to determine if the optical properties of the carbon film were suitable for an ellipsometric investigation of protein adsorption on this material. IBD carbon coatings have been termed "diamond-like" because of their durability in certain applications and because their refractive indices apparently approach diamond rather than graphite (15). If the carbon coating were truly "diamond-like"

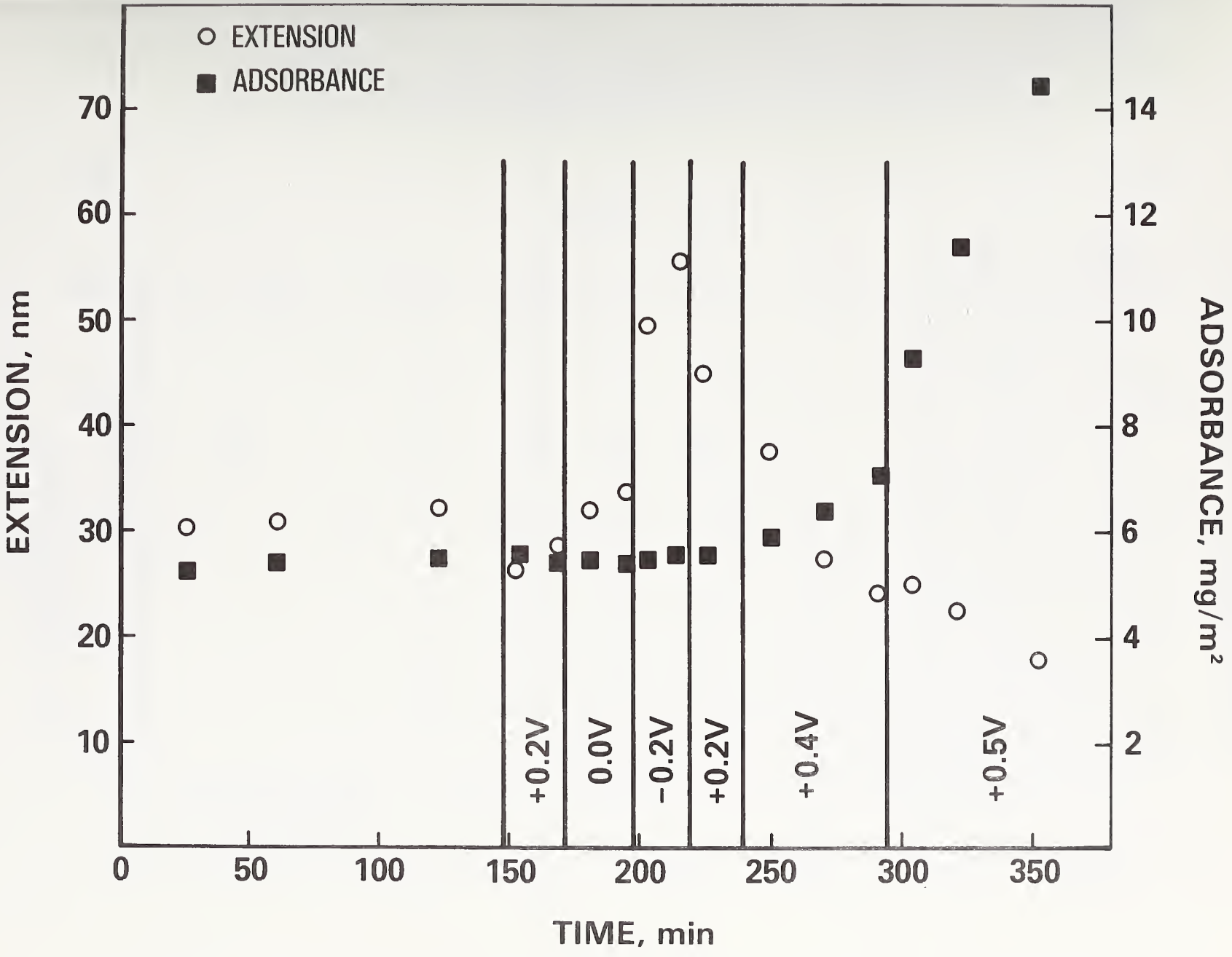


Figure 1: Best fit extension and adsorbance values vs. time for human fibrinogen at 3.2 mg/ml and pH 7.32 adsorbed on platinum. Applied surface potential indicated between vertical bars.

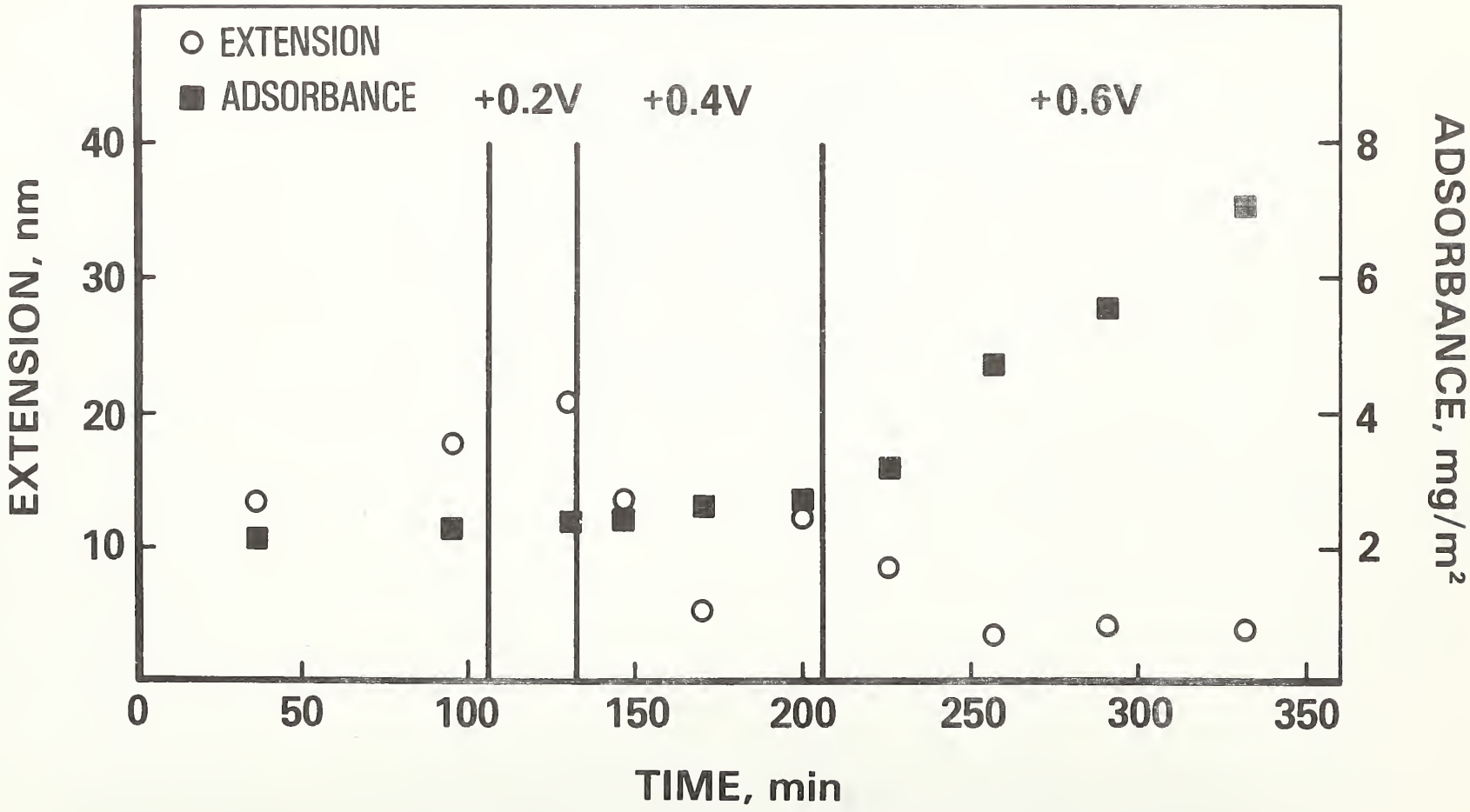


Figure 2: Best fit extension and adsorbance values vs. time for human serum albumin at 5.0 mg/ml and pH 7.45 adsorbed on platinum. Applied surface potential indicated between vertical bars.

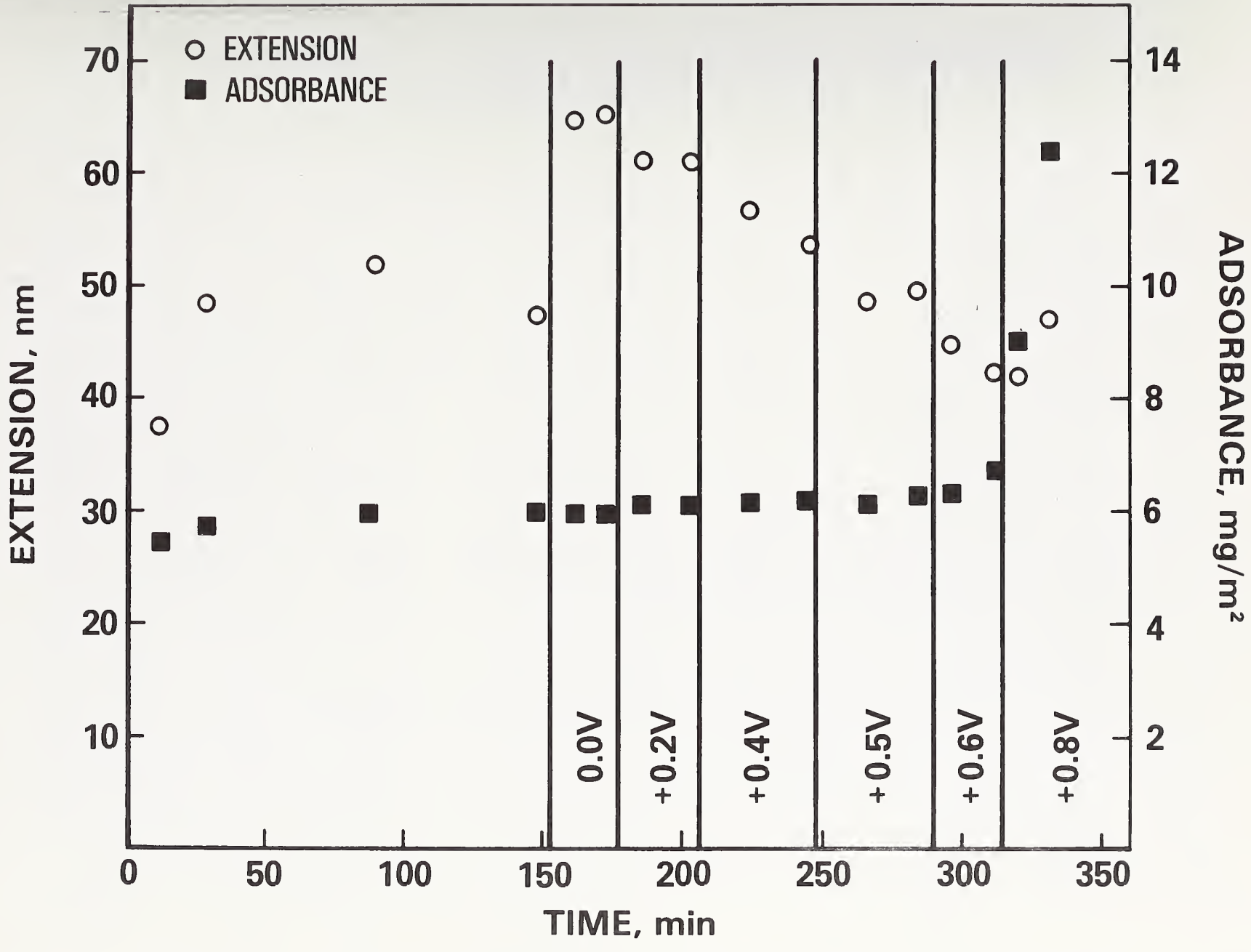


Figure 3: Best fit extension and adsorbance values vs. time for bovine γ-globulin at 11.4 mg/ml and pH 7.32 adsorbed on platinum. Applied surface potential indicated between vertical bars.

Table I: Effect of Surface Potential on Protein Adsorption

Protein	Conc. mg/ml	Potential Range, V/SCE	Adsorbance at Rest Potential mg/m^2	Extension at Rest Potential nm	Critical Potential V^*/SCE
Fibrinogen	2.5	-0.2 to +0.5	6	30	+0.4
Serum Albumin	5.0	+0.2 to +0.6	2	10	+0.6
γ-Globulin	10.0	0.0 to +0.8	6	45	+0.8

it should have a high index of refraction and very small optical absorption at visible wavelengths. Both of these properties would be desirable for ellipsometric applications.

In order to measure an adsorbed protein film, the optical properties of the substrate must be known accurately. This generally entails the measurement of the refractive index of the substrate (both real and complex components) immediately before the surface is covered with protein. In the case of a composite substrate, this procedure is not sufficient and additional data must be supplied. If the optical constants (refractive index) of the uncoated substrate are known, then the thickness and the real refractive index of the carbon coating could be measured ellipsometrically. The composite substrate would then be completely specified if the carbon film were non-adsorbing.

IBD carbon films on silicon and polyurethane (Stanford Research Institute sample 3-425-1-X) were measured ellipsometrically. Samples which were not beam scanned had a non-uniform film thickness and were not measurable. Beam scanned samples proved satisfactory. Silicon was chosen as a substrate because sample to sample variation in the optical constants of silicon have been found to be relatively small so that the refractive index of uncoated silicon samples could be used in the calculations necessary for the IBD coated measurements. Only two variables are measured by ellipsometry so that only two parameters of a single film can be measured simultaneously. By measuring the same film on two different substrates, however, another parameter can be specified. From these measurements it was concluded that IBD carbon coatings have significant optical adsorbance at 632.8 nm. The measured thicknesses correspond very well with those estimated by Whittaker Corp., but would not be consistent with a high real refractive index and a low complex part. Theoretically, protein adsorbance measurements could still be made under these conditions, but the experiments would be involved and additional sources of errors would be introduced.

Low Temperature Isotropic

Prothrombin adsorption on LTI carbon supplied by General Atomic Corp., both silicon alloyed and unalloyed, has been measured ellipsometrically. A highly purified prothrombin solution supplied by Prof. Craig Jackson having a concentration of 0.22 mg/ml, yielded an adsorbance of 2.9 mg/m^2. The thickness measurements did not vary appreciably with time and were quite reproducible from sample to sample. The range of extensions observed varied from 4.5 to 8.7 nm with an average value of 6.2 nm. These values are significantly smaller than those observed on other substrates and the differences are not due to the high purity protein used for the carbon. For example, an average extension of 16 nm was measured for the purified prothrombin on chrome compared with 22 nm measured using commercially available protein. The significance of this low extension must be compared with the observation of very inhomogeneous albumin adsorption on this carbon reported in the radiotracer section of this report. The effect of such possible surface porosity on ellipsometric measurements would be expected to be small, but definitive answers must await a further investigation of the causes of such high local protein concentrations.

BOUND FRACTION

Surface Concentration Induced Conformational Changes

Speculation based on indirect evidence (16,17), as well as analogies with protein properties at the air-solution interface, have frequently led to the assumption that all proteins must "denature" or change drastically in conformation upon adsorption at the solid-solution interface. In previous studies utilizing serum albumin and prothrombin (18,4), the molecular conformation determined from bound fraction measurements was unaffected by variations of surface concentration along the adsorption isotherm. In order to clarify this extremely important aspect of protein adsorption, bound fraction and ellipsometric studies have been carried out on the adsorbed conformation of β-lactoglobulin and γ-globulin. These proteins were selected on the basis of previous infrared studies of monolayers by Loeb (19) and the potentiometric titration results of Kochwa and coworkers (20), both of which indicated that changes in conformation paralleled changes in the surface concentration.

The adsorption isotherms of native β-lactoglobulin and γ-globulin adsorbed on silica at pD 7.4 are shown in Figures 4 and 5. The values given along the curves represent the experimentally measured bound fraction (the fraction of carbonyl groups of an adsorbed protein molecule directly interacting with the surface) at that equilibrium concentration. In each case, it is evident that there is a significant decrease in the bound fraction with increasing adsorbance. For β-lactoglobulin, the decrease in bound fraction from 0.14 to 0.01 corresponds to a decrease from 30 to 2 carbonyl surface attachments per molecule, while for γ-globulin, the change of bound fraction from 0.2 to 0.02 results in a decrease from 270 to 30 contacts.

To further investigate these changes in conformation of γ-globulin and β-lactoglobulin, we have determined the extension of the adsorbed film on planar fused silica using ellipsometry. The protein solution concentrations were selected to correspond to those regions of the adsorption isotherms (Figures 4 and 5), which exhibited the largest differences in the bound fraction. As shown in Table II, the extension of adsorbed γ-globulin increased with increasing concentration of the solution. At concentrations of 0.11 mg/ml and 10.0 mg/ml, bound fractions of 0.16 and 0.02, respectively, were measured for the adsorbed γ-globulin, while extensions of 19 and 64 nm were observed, respectively. Similar ellipsometric studies utilizing β-lactoglobulin were unsuccessful. We can only spectulate that the relatively small size of the β-lactoglobulin molecule does not result in a sufficient difference in index of refraction to define the adsorbed layer.

The decreasing bound fraction (number of surface attachments) of γ-globulin with increasing adsorbance has been reported also for a number of synthetic, random coil polymers. Using infrared bound fraction measurements to study the adsorbed conformation of poly(methyl methacrylate) and polystyrene on silica, Thies (21) observed a decreasing number of surface attachments with increasing

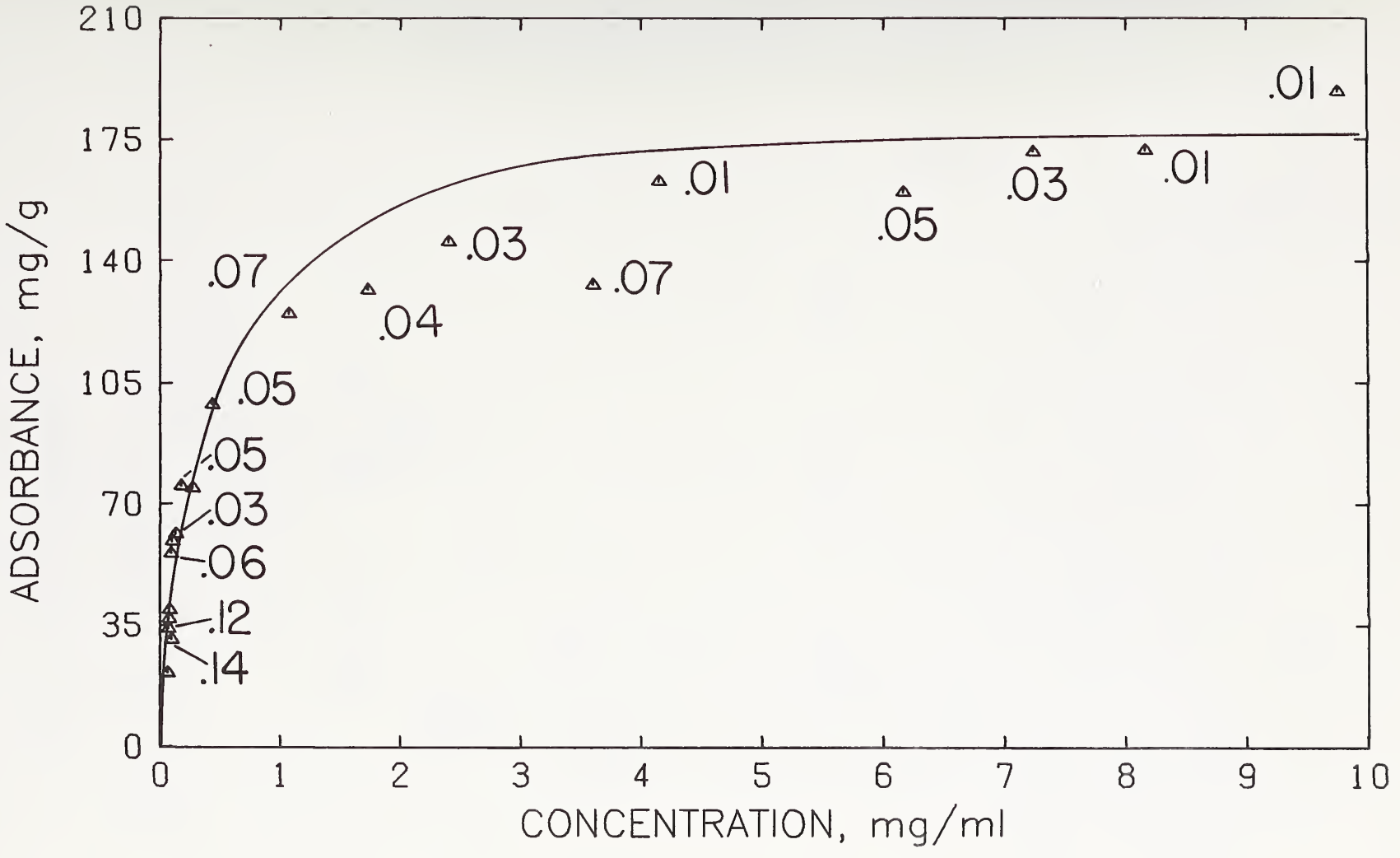


Figure 4: Adsorption isotherm for bovine β-lactoglobulin at pD 7.4 on silica. Values of bound fraction given for isotherm points.

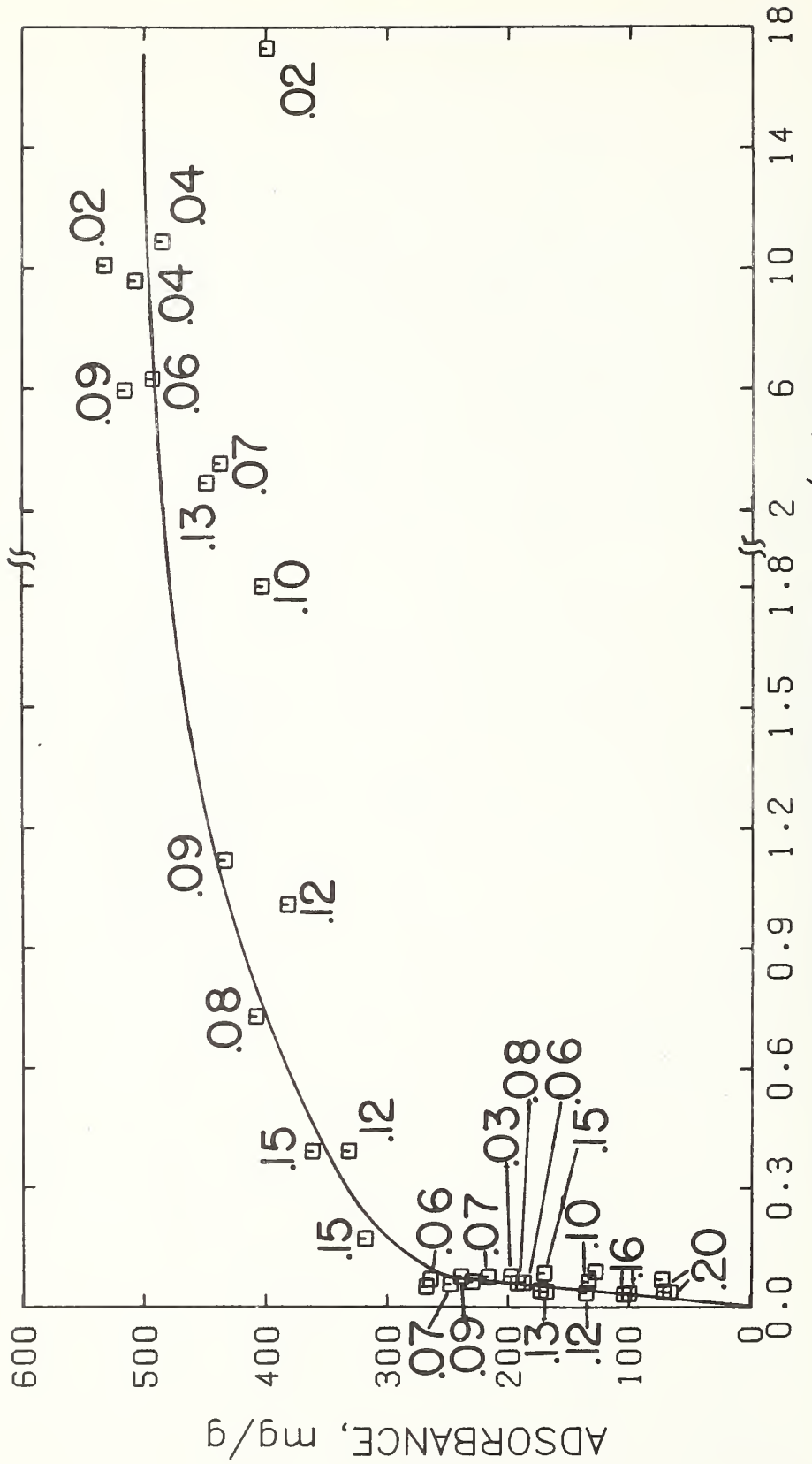


Figure 5: Adsorption isotherm for bovine γ-globulin at pD 7.4 on silica. Values of bound fraction given for isotherm points.

Table II

Extension of Adsorbed γ-Globulin

Native

Conc. mg/ml	Extension nm	Bound Fraction
0.11	19	0.16
0.20	32	∿0.12
0.28	27	∿0.12
10.0	64	0.02

DEM Crosslinked

0.30	21	0.06
10.7	20	>0.01

adsorbance. Similarly, Fontana (22) showed that the bound fraction of an alkyl methacrylate-polyglycol methacrylate copolymer decreased with increasing adsorbance. The ellipsometrically measured extension of an adsorbed molecule normal to a surface has been found to increase with time during the adsorption period for the adsorption of polystyrene (23,24) and polyethylene glycol (25). The increase in extension, as well as the decrease in the bound fraction, with increasing surface concentration have been interpreted to result from a decrease in the number of surface attachments. The similarity of these results to those reported for γ-globulin and β-lactoglobulin suggests that both proteins undergo conformational changes. The agreement between changes in bound fraction and extension for adsorbed γ-globulin supports the proposed conformational change.

The similarity of the results for synthetic polymers and γ-globulin implies that the protein is rather pliant. Biologically, this feature is undoubtingly important, since in solution, 7S γ-globulin is an important antibody which must interact with a wide variety of antigens. Furthermore, recent studies by Kim and coworkers (26) have shown that prior adsorption of γ-globulin results in the significant adhesion of platelets to a surface. Such effects may indeed depend on the adsorbed conformation.

The bound fraction and ellipsometric conformation studies of β-lactoglobulin and γ-globulin are entirely consistent with previous findings (19,20). Loeb demonstrated a change in the ratio of the intensity of the infrared bands characteristic of α-helical and β-sheet structures for β-lactoglobulin in a transferred film as a function of surface pressure in the spread monolayer. This finding was interpreted as a "denaturation" at low surface concentration, although the introduction of artifacts due to drying and working with collapsed films make it difficult to establish whether the molecule was folding or unfolding as it "denatured". Kochwa's in situ potentiometric titration study of the adsorption of γ-globulin to a polystyrene latex showed that additional titrable groups per adsorbed molecule appeared at low surface concentration. The strong correlation between surface concentration and acid uptake established a definite change in conformation upon adsorption as a function of the adsorbance.

None of these results (potentiometric titrations, infrared spectroscopy of transferred monolayers, ellipsometric, or bound fraction) for native γ-globulin and β-lactoglobulin actually establish at what point along the adsorption isotherm the adsorbed protein is "native". While analogies to air-liquid adsorption studies where the low concentration, low pressure state coincides with a spread monolayer have frequently been made, studies of polyelectrolytes adsorbed at the solid-liquid interface (27) establish that adsorbate-adsorbate interactions generally determine both the adsorbance and conformation. It is therefore possible that major distortions of the native protein conformation could occur at high, rather than at low, adsorbance.

To further investigate these changes in γ-globulin conformation, as well as the question of which adsorbed conformation corresponds to that in solution, bound fraction and ellipsometric studies were carried out using the cross-linked protein. Diethyl malonoimidate hydrochloride (DEM) was reacted with γ-globulin as previously described (18) to block 56% of the

free amino groups (see Experimental section, p. 3).

The adsorption isotherm of cross-linked DEM-γ-globulin is shown in Figure 6. The values given along the curve represent the experimentally measured bound fraction at that equilibrium concentration. Compared to native γ-globulin (Fig. 5), there are approximately one half the number of carbonyl attachments at a given equilibrium concentration. The extension of the adsorbed cross-linked protein is given in Table II at two concentrations approximately equal to those used to study native protein. At 0.30 mg/ml and 10.7 mg/ml, the extensions are experimentally indistinguishable from each other and closely resemble the results for low concentration native protein. Thus, while the bound fraction results indicate that cross-linked γ-globulin is still somewhat flexible, with an adsorbed conformation intermediate between those at high and low surface concentrations, the ellipsometric results indicate that the conformation at low surface concentration is that of the native protein. It would appear that if the cross-linking reaction is "freezing" the native, solution conformation, then the native conformation exists at low surface coverage and the "denaturing" force is the protein-protein interactions between adsorbed molecules.

Caution must always be exercised when comparing bound fraction and ellipsometric results for different proteins. It is difficult to establish correlations between the techniques and draw conclusions concerning conformational changes of adsorbed proteins. Native and cross-linked γ-globulin yield similar extensions at low concentration, despite the significant difference in bound fraction. It is quite conceivable that this could occur, since the introduction of additional constraints on internal motion would certainly limit local rearrangements in the vicinity of the surface, without changing the overall molecular dimensions. Only for a flexible, random-coil polymer would one expect in general a correlation between the number of surface attachments and the extension. Even in this case, however, unusual adsorbed conformations could destroy the correlation.

Surface Attachments on Hydrophobic Surface

A monodisperse polystyrene latex of 91 nm particles (63 m^2/g) was purified using mixed ion-exchange resins (28) and techniques developed for its use as an adsorbent for infrared bound fraction measurements. Since all adsorption suspensions must be prepared by pipetting protein solution and latex, the errors inherent in the procedure are somewhat greater than in the case of silica.

The results of experiments to date define an isotherm for bovine serum albumin with an apparent plateau adsorbance of 140mg protein per gram of polystyrene latex at an equilibrium concentration of ∿2.0 mg/ml. This adsorbance is some 2.5 times greater per unit area than that occurring on silica. The values of the bound fraction, while generally higher than on silica, show a large scatter. Efforts are in progress to refine the technique and reduce the uncertainties.

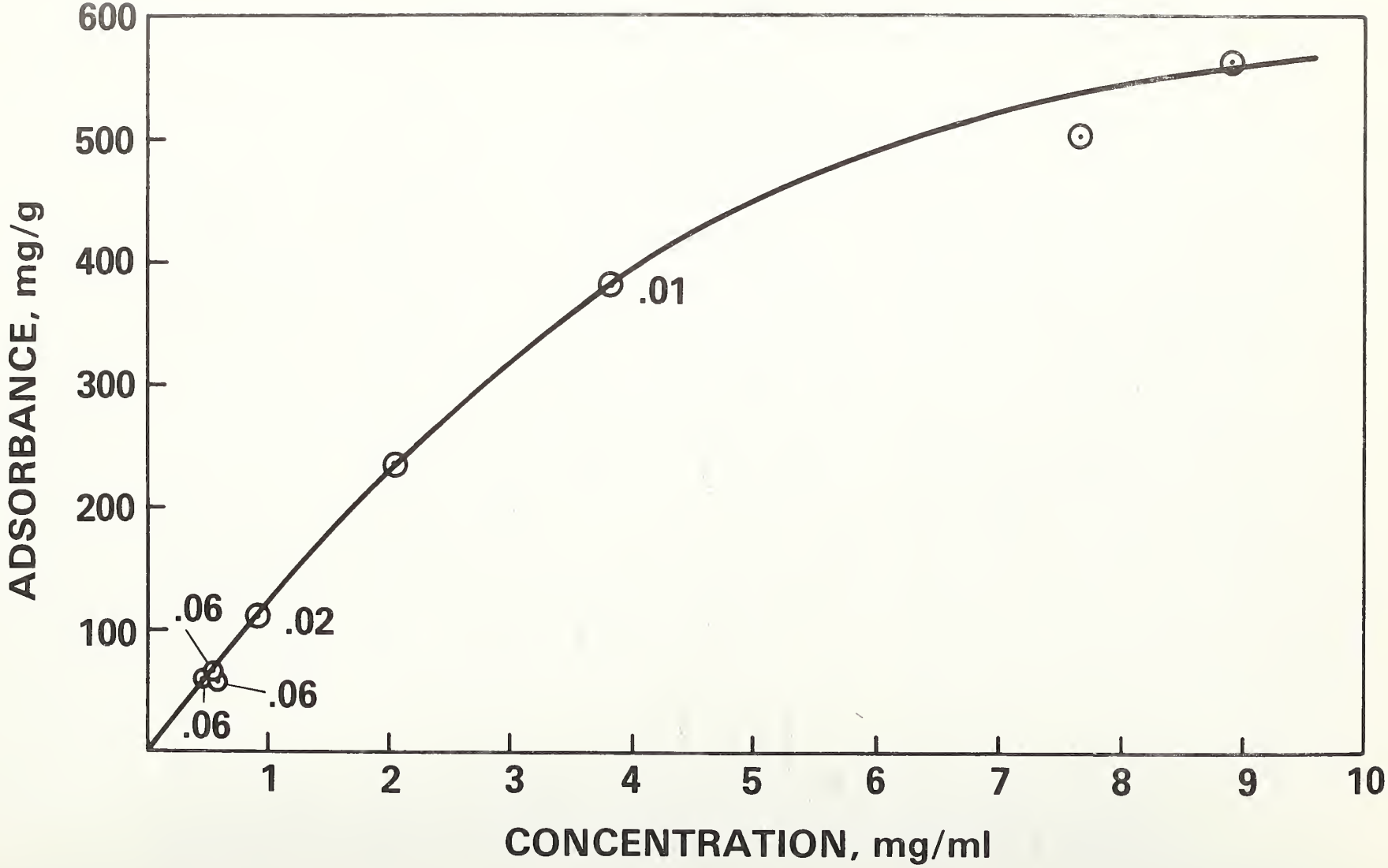


Figure 6: Adsorption isotherm for γ-globulin cross-linked with diethyl malonoimidate hydrochloride. Values of bound fraction given for isotherm points.

RADIOTRACER RATE STUDIES

The success of an investigation of the rates of adsorption, desorption and exchange of blood proteins is dependent, in part, on the development of techniques that will be effective in completely removing any "carry-out" from the adsorption solution and will prevent coating the slide with denatured protein that may exist at the solution-air interface. This problem and the techniques employed here were discussed in some detail in our last annual report (4). An unexpected problem, preferential or specific adsorption of the labeling atom, was also observed for platinum, one of the materials investigated. This problem has been investigated somewhat further and is discussed below. Finally, rates of adsorption for human serum albumin on chromium and silica, shown in our last report are not given again here, but are used as the basis for our desorption and exchange results.

Silica

The rate of desorption of albumin into water from fused silica is shown in Figure 7. A very rapid initial rate of desorption occurs; about 30 to 35% of the adsorbed material is removed within approximately one minute. Subsequently, there is no further change in the amount of protein remaining for time periods up to two hours. The desorption of albumin into buffer, as seen in Figure 8, however, indicates a somewhat different behavior. A similar rapid initial desorption is also observed, although it appears to continue for a longer time period with more protein removed. A second, slower, desorption rate also occurs over longer time periods. In this case approximately 50% was desorbed within the first five minutes, with an additional 30% removed in the subsequent four hour period. The second rate may be a function of the ionic strength of the solvent.

Also investigated was the rate of exchange of adsorbed (labeled) albumin with albumin (nonlabeled) in solution. Under the conditions of the experiment, the total quantity of albumin on the surface should be constant. Shown in Figure 8 is the amount of initially adsorbed albumin, that remained on the surface. This is seen to be, within experimental error, the same as the amount remaining after desorption into buffer for the system and time period investigated. The similarity between rates of desorption and exchange were also observed for a random coil synthetic macromolecule, polystyrene (29).

Polyethylene

Measurements of HSA-^{131}I adsorbance on polyethylene as shown in Figure 9, show that the apparent equilibrium adsorbance is not reached for 15 min. compared to 10 sec. for chrome and 15 minutes for silica reported previously (4). The differences in rates to plateau values may be related to the surface free energy, i.e., as the surface energy decreases, the times to plateau decrease. An increase in the extension with decreasing surface energy was noted for several blood proteins (4).

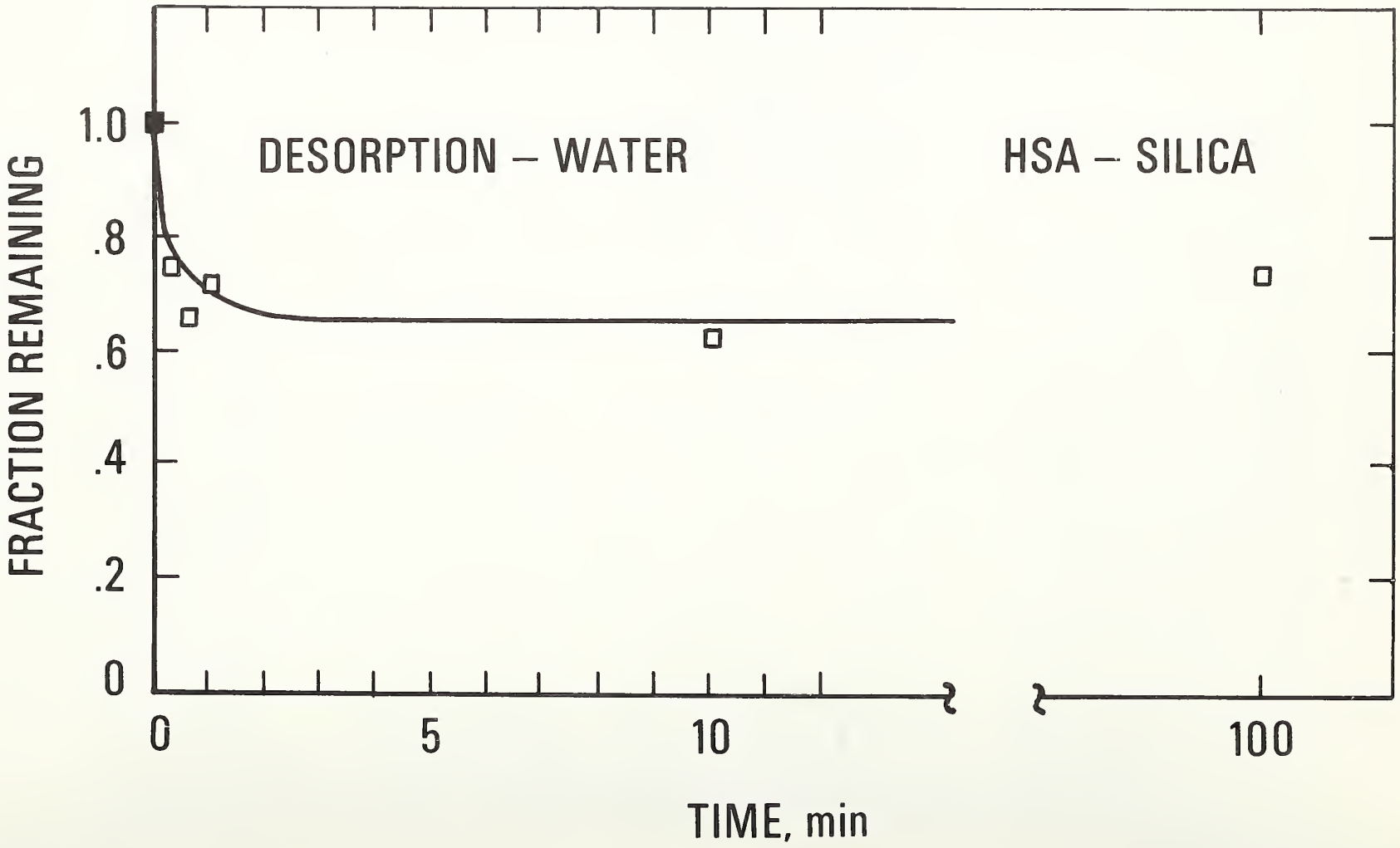


Figure 7: Desorption of serum albumin from silica

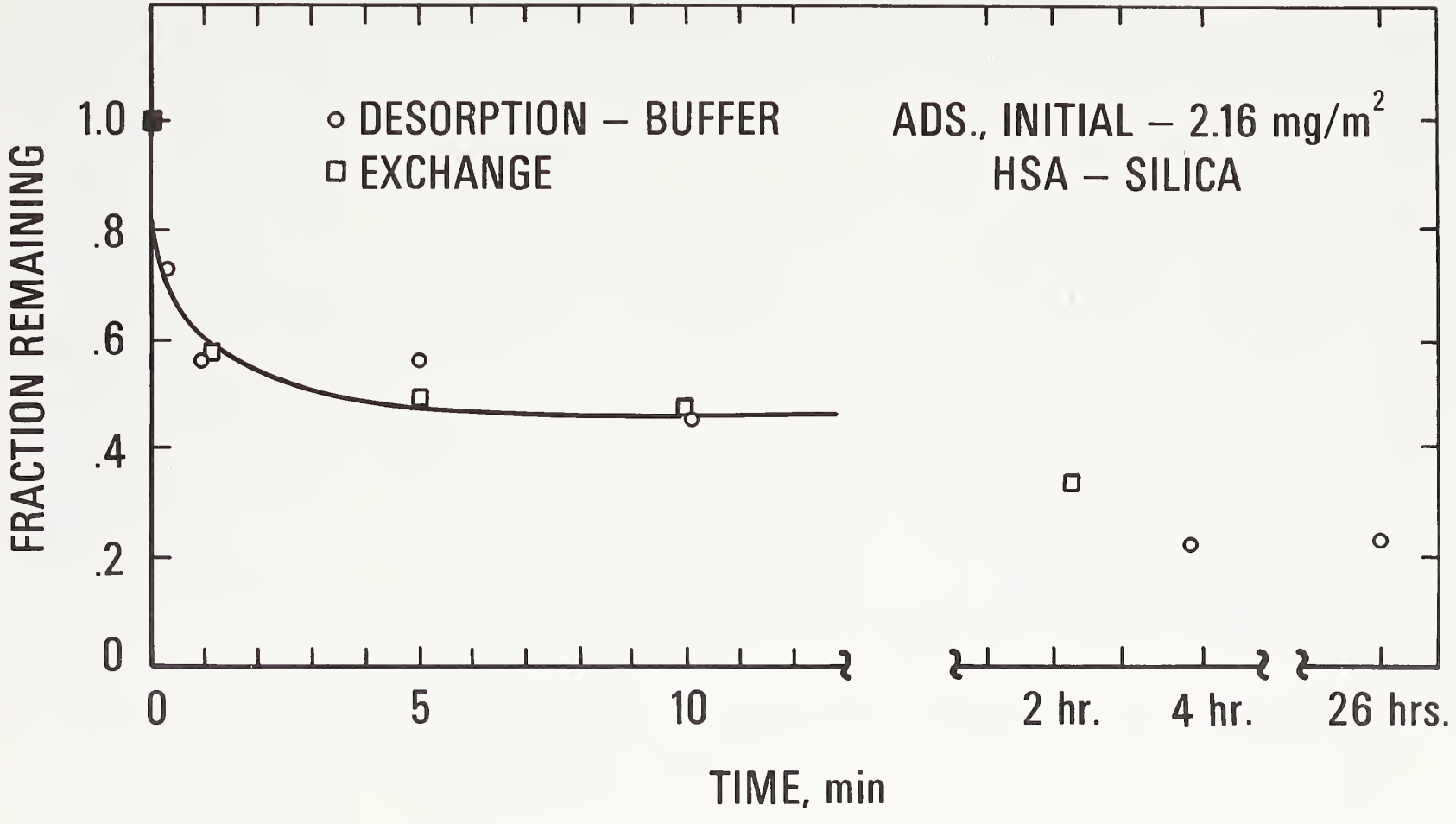


Figure 8: Desorption and exchange of serum albumin from silica

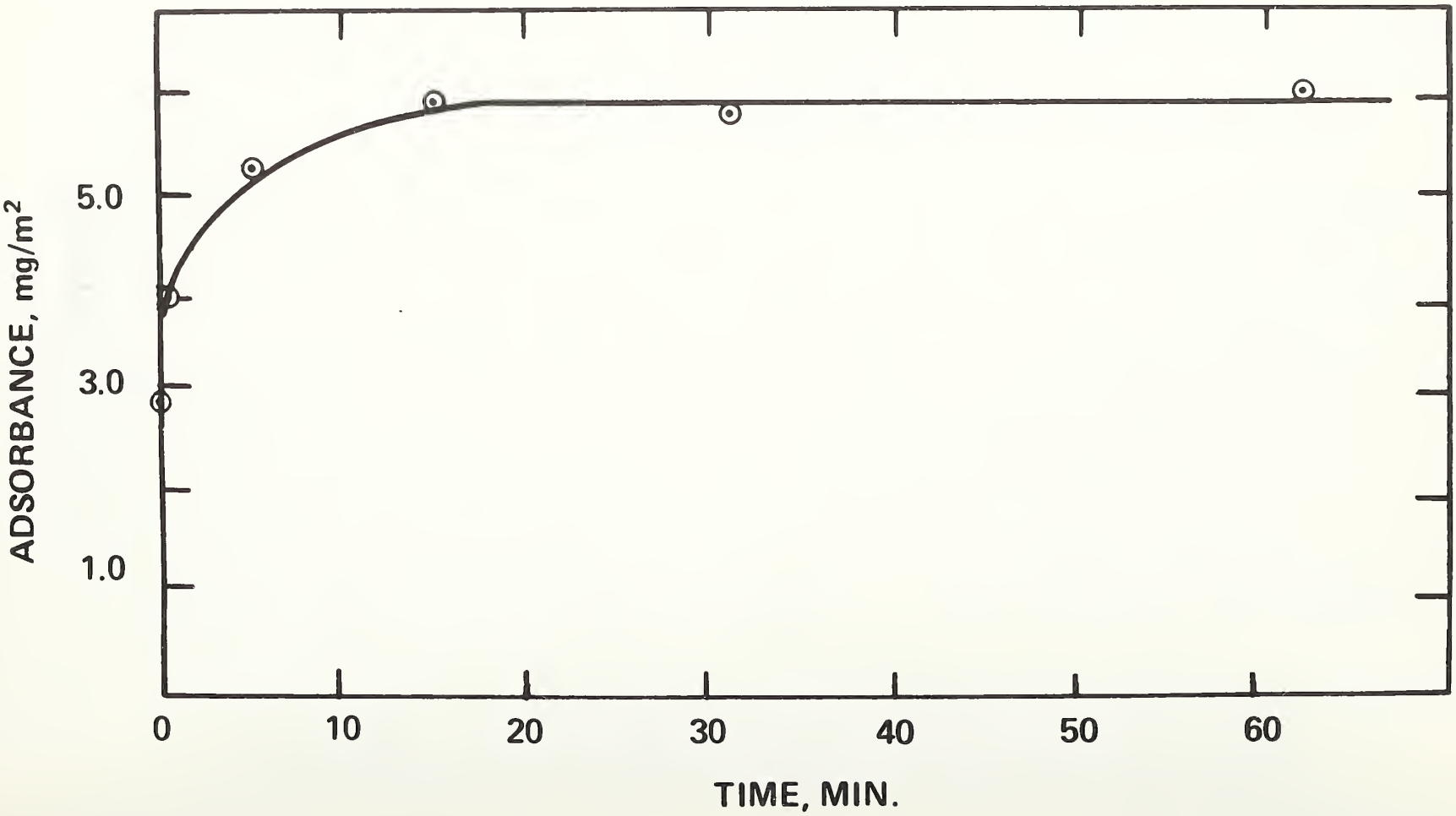


Figure 9: Rate of serum albumin adsorption on polyethylne.
Solution concentration 5.0 mg/ml.

Platinum

In our last report (4) we discussed the abnormally high apparent adsorbance values obtained on platinum with HSA-^{131}I and described the results of the use of solutions of different activities of ^{131}I to test for its adsorption. Additional work has verified that platinum interacts with ^{131}I to give erronous results when this atom is used to label the protein. Other materials were also investigated to determine whether they were also subject to specific adsorption of ^{131}I. The results are shown in Table III for three polymeric materials. Unlike the platinum, the adsorbance values are independent of the specific activity for these three polymers. Similar results were obtained for silica and chrome.

Comparison was made of adsorption on platinum (and chromium as a reference) between HSA-^{131}I and albumin labeled with tritium-containing acetyl groups. As can be seen in Table IV, the chromium measurements agreed well, but the adsorbance measured by the tritiated albumin on platinum was less than 5% of the apparent adsorbance value obtained using the iodinated albumin. This is a fairly direct conformation of our hypothesis of enhanced specific adsorption induced by the incorporation of iodine into the albumin.

Low Temperature Isotropic Carbon

The adsorption of HSA^{131}I on low temperature isotropic carbon (LTI) has been studied. All results were obtained using the same experimental procedure as outlined in the previous section. An initial study showed an equilibrium adsorbance of 12.9 mg/m^2 on the carbon surface, but only about 3 mg/m^2 was removed by extensive scrubbing with detergent solutions. This same scrubbing treatment has generally been successful in removing all of the adsorbed albumin from other surfaces, such as quartz and platinum. This suggests that porosity of the surface might be suspected as being responsible for both the rather high adsorbance values and the difficulty in desorption. Porosity of the surface might arise from a generalized homogeneous distribution of vacancies on the scale of atomic or crystallite dimensions or, alternatively, porosity might be centered in defect structures of a larger scale and would be distributed inhomogeneously across the surface. Existing information about the LTI carbon indicates that the first possibility is somewhat remote, thus attempts were made to experimentally evaluate the possibility of inhomogeneous adsorption associated with larger scale porosity.

Figure 10 shows a typical radiograph where the dark spots indicate a high concentration of serum albumin. The small white spots are pin holes in the film and the large white area in the lower right is a portion of the film that was not in contact with the surface and, therefore, serves as a blank. Figure 11 shows a larger portion of an exposed film for a second carbon sample of the same lot under similar experimental conditions. The general gray background indicates a rather homogeneously distributed component of the adsorbed protein that is present on all areas of the carbon surface and was not removed by the detergent washing. That this gray background is due to radiolabeled protein and not light exposure or development fog is seen by comparison with the white area in Figure 10. The very dark spots

Table III. Dependence of Adsorbance on Specific Activity of HSA-^{131}I

Relative Activity	Polyethylene mg/m^2	Silicone mg/m^2	Polyethylene Terephthalate mg/m^2
1.00	5.6	6.1	6.0
0.67	4.4	7.2	5.4
0.50	5.4	6.5	5.7
0.33	4.7	6.5	6.1
0.25	5.3	5.2	5.6
0.20	4.6	-	5.7

Table IV. Adsorbance of Albumin, mg/m^2, Estimated using Different Radioactive Labels

	Pt. mg/m^2	Cr. mg/m^2
H^3-acetyl group label	3.56	3.75
^{131}I label	77.0	2.67

scattered across the film indicate that a large fraction of the measured adsorbance is associated with very high protein concentrations in small areas. Enlargements of several selected areas of the film that include some of these very dark areas are shown in Figures 12-15. The structure in these areas is striking in that many seem associated with surface scratches or cracks. This is especially apparent in Figures 14 and 15 by the "beads on a string" appearance of the autoradiograph, suggesting that sub-surface porosity may have been opened up by a scratch or crack.

The carbon surface underlying one of the radiographs is shown in Figure 16 at the same magnification as the film seen in Figure 10. At this level the surface seems more or less homogeneous with small hills and depressions and a few concentric rings. Alignment of the film with the surface from which it was stripped showed no correspondence of the dark spots due to high proteins concentration with these concentric rings. Higher magnification of the carbon surface are shown in Figures 17 and 18. The magnification in Figure 17 corresponds rather closely to that of the autoradiographs in Figures 12-15. While the carbon surface is covered with numerous scratches and cracks at this scale, they are much smaller than the features which are disclosed by the protein adsorbance. Further autoradiographs will be taken that will include reference marks to permit exact alignment of the film with the surface. This should permit a better identification of the surface features responsible for the high protein concentration.

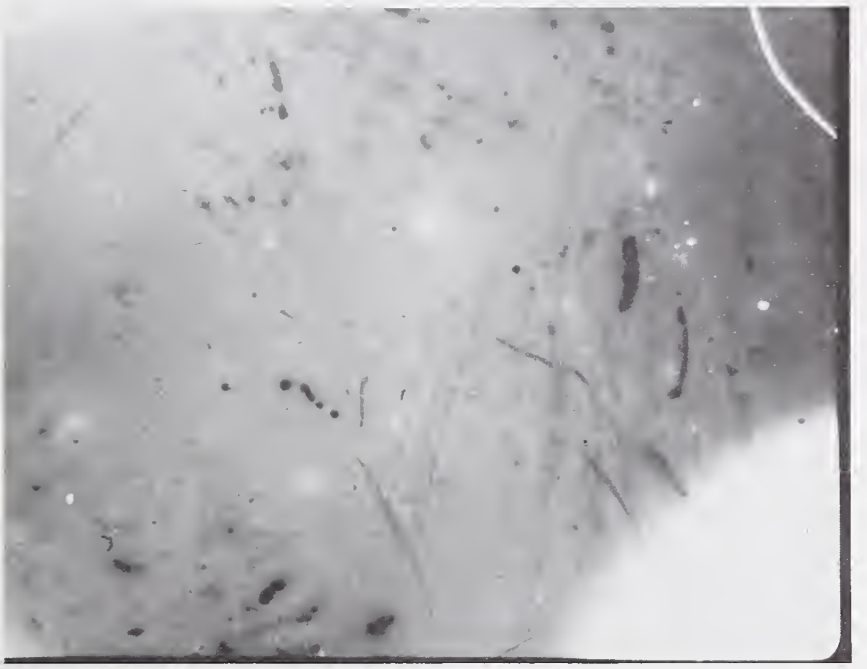


Figure 10: Autoradiograph of HSA-^{131}I on LTI carbon

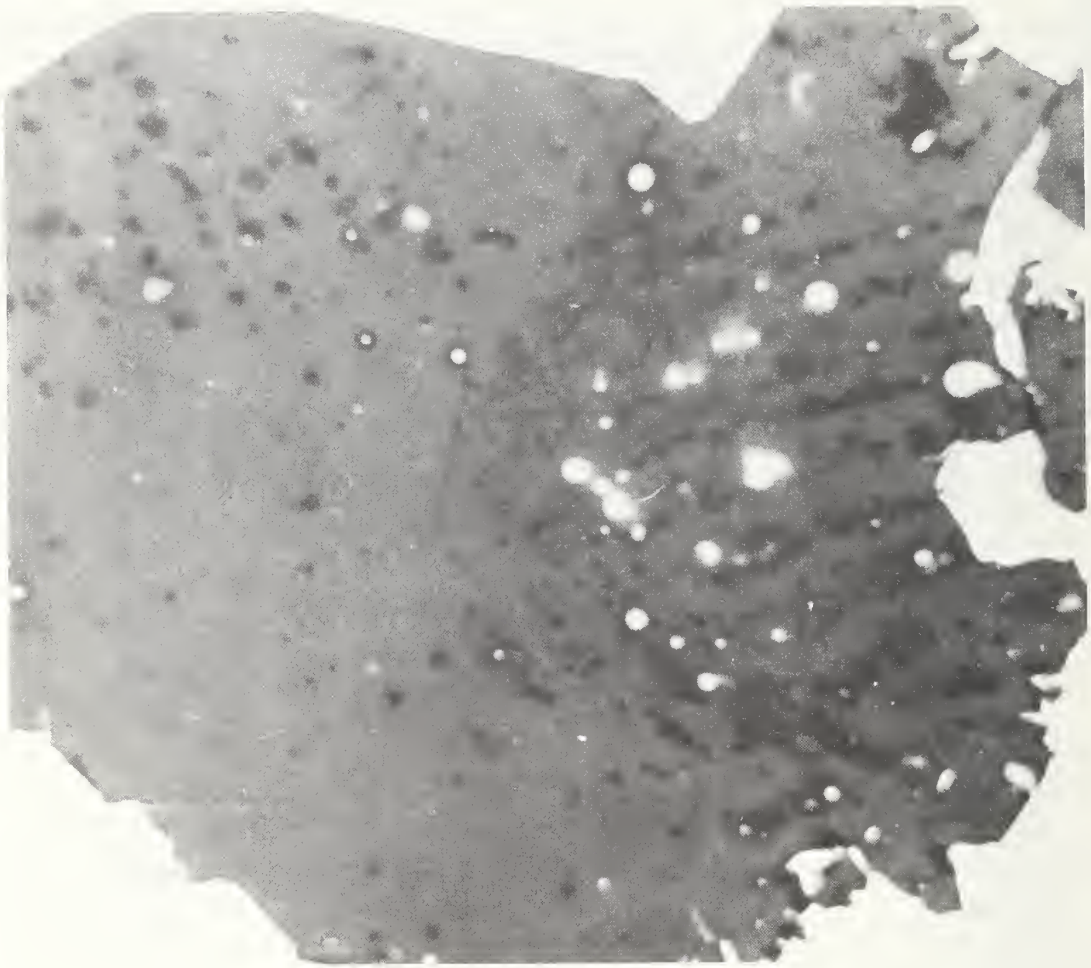


Figure 11: Autoradiograph of HSA-^{131}I on LTI carbon

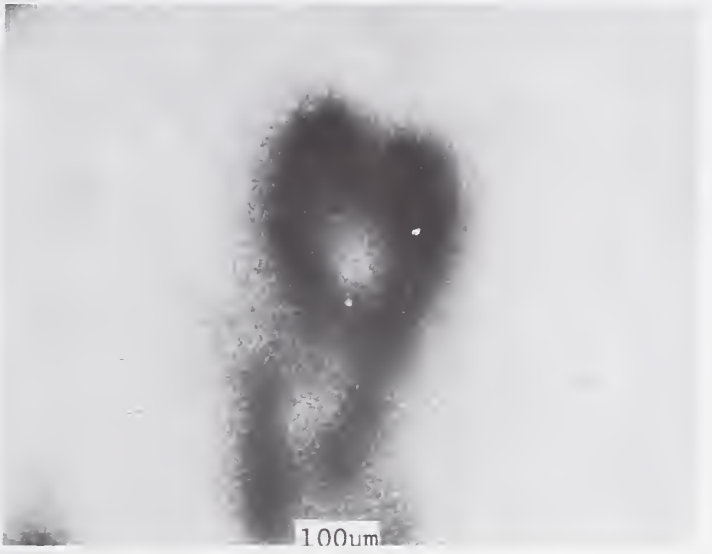


Figure 12: LTI carbon surface

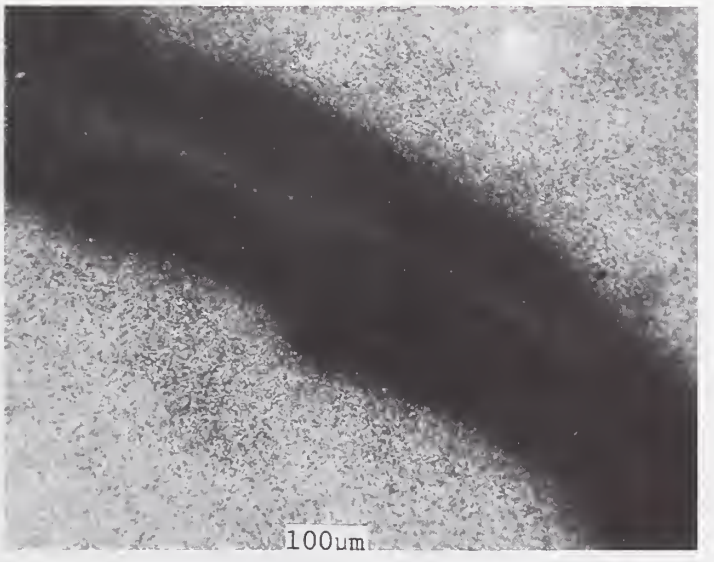


Figure 13: LTI carbon surface

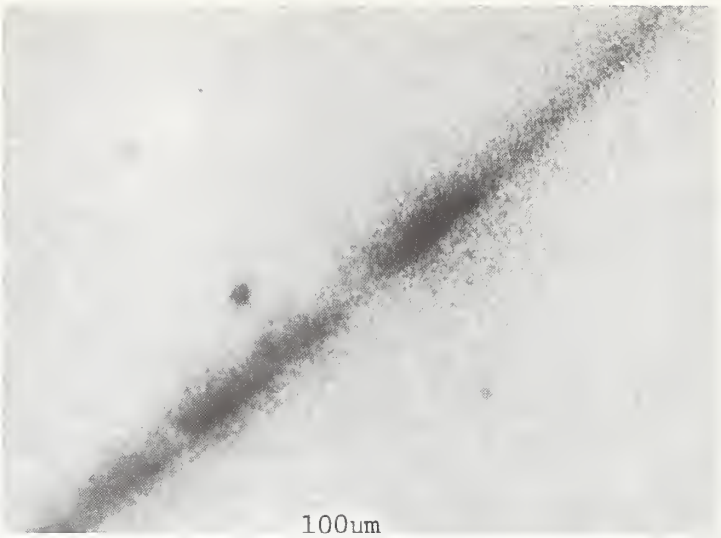


Figure 14: LTI carbon surface

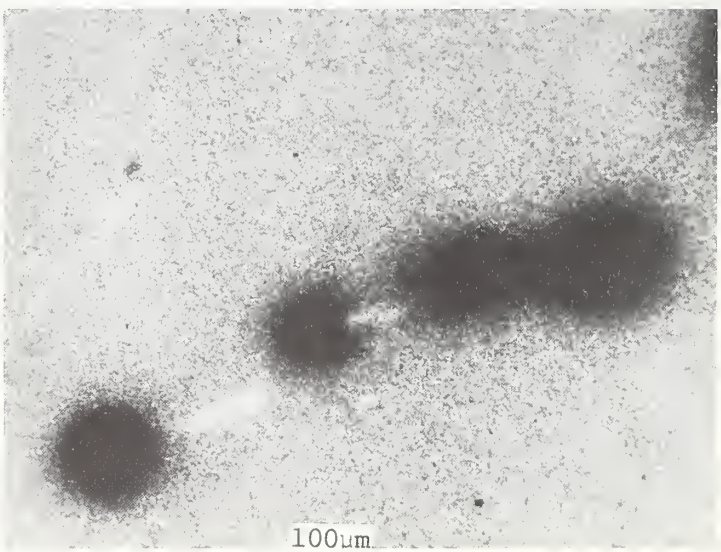


Figure 15: LTI carbon surface

Figure 16: LTI carbon surface

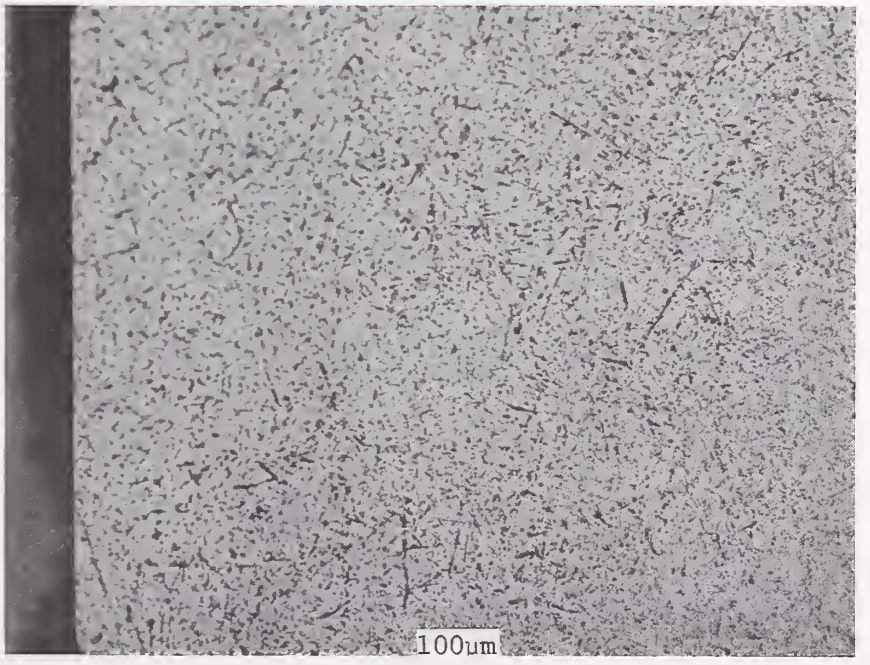
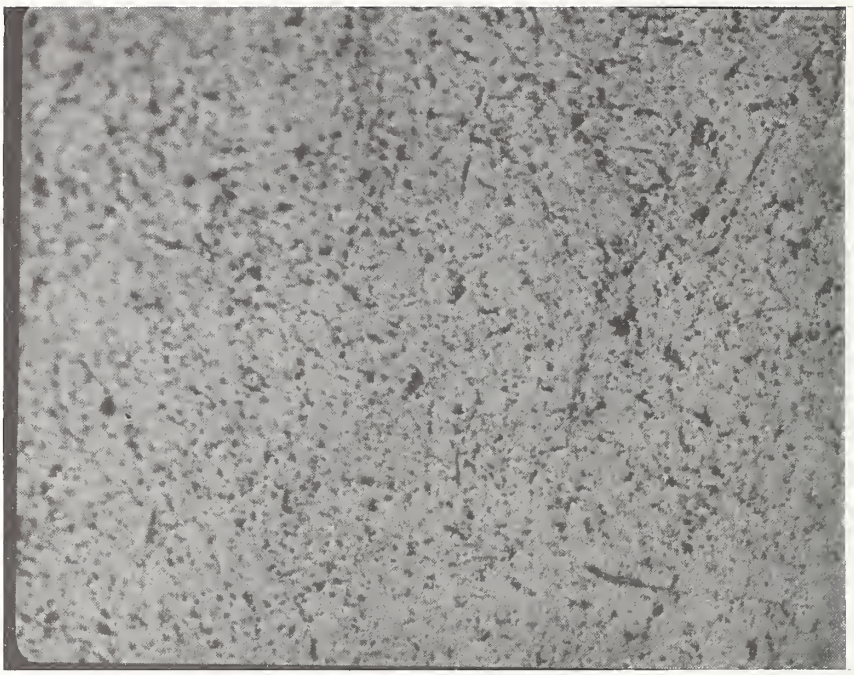


Figure 17: LTI carbon surface



100 μm

Figure 18: LTI carbon surface

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